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STAIN TECHNOLOGY

OCTOBER, 1926

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STAIN TECHNOLOGY

VOLUME I OCTOBER 1926 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

STANDARDIZED STAINING TECHNIC

Is standardization of staining technic possible? Or if possible, is it desirable? The answer to both questions is certainly negative if by standardization is meant the reduction of all staining procedures to cut-and-dried routine that must be followed alike by the untried novice and by the brilliant technician who appears to have a special genius for getting results obtainable by no one else. Used in another sense, however, a certain degree of standardization is desirable, and perhaps possible.

To illustrate the point, let us suppose that John Smith works out a brilliant and complicated staining technic for showing some special histological or cytological structure. It is published; and from his photomicrographs, drawings, and careful descriptions the importance of the technic is evident to many who do not think of trying to duplicate the results. It is hailed with much acclaim as the John Smith technic. Meanwhile a few of John Smith's students learn the details of the technic under his instruction; and when they go to other institutions they teach the method to students of their own. Also some of John Smith's own generation pick up the technic while visiting in his laboratory. And, lastly, others try the method by following the published account of it without seeing the procedure carried out either by its originator or by one of his students. The technic is thus adopted in quite a number of laboratories, and is known by the name of John Smith.

Now, it is a well-known fact that a complicated staining procedure, involving the use of two or more stains, cannot be written up so that anyone else can follow it exactly and get the proper results, unless he also is a good technician and can adapt the procedure to his conditions as he goes ahead. This being the case, there is a good chance that the John Smith method may be unconsciously modified and give quite different results in one laboratory from what it does in another.

Furthermore, John Smith's own slides may not look so clear and sharp to another as they do to himself. It is obvious that a person who is color-blind would have difficulty in picking out the minute details in a preparation made by a delicate double or triple staining procedure. It is not so obvious, however, that a person with sufficient

color perception to get along perfectly in every-day life might be sufficiently lacking in the perception of one of the primary colors to fail to see something in these slides perfectly visible to John Smith. In recent years, when the colorimetric determination of hydrogen ion concentration has come to play a large part in the chemical and bacteriological laboratory, it has become evident that some laboratory workers can obtain sharper readings with the indicators that give greens and blues; others with those that turn thru orange into red; and yet none of the individuals in question are noticeably color-blind.

Let us suppose that John Smith's eyes are especially keen for reds, and that his technic calls for a distinction between tissue elements that are stained red and orange respectively. Then when John Doe, whose eyes are more sensitive to the greens and blues, tries the method, he may get results identical with those of the author, but looking different to him; and he realizes that he cannot see all that John Smith described. If he is a good technician, and is interested in the method, he suspects that his technic is at fault, and tries modifications until he hits on a procedure that makes them slightly purple; then with the slightly bluish shade in the tissue elements thus stained he can immediately distinguish them from the orange structures. Should he show these slides to John Smith, however, the latter might not like them because to his eyes the purple appears too much like the bluish shades shown by other structures. In all probability, however, Smith and Doe never get the chance to compare these preparations, and Doe continues to think he is using the Smith technic without any important modification.

A still further possibility of modification in the original technic may occur from similar reasons. John Smith may continue using the technic as he grows older and his eyes become less keen. What would happen? Probably, just as our grandfathers, before the days of eyeglasses to correct optical defects, used magnifying glasses to increase the visibility of print, so would John Smith increase the visibility (to him) of the microscopic preparations by staining them more deeply. Assuming, as we have done, that his eyes were always more sensitive to reds than to greens and blues, he would particularly try to increase the depth of the latter colors in his sections. If his laboratory force were made up mostly of technicians whose object was rather to prepare slides acceptable to their chief than to make preparations adapted to their own eyes, the result might be that the technic would yield results in its author's laboratory appearing quite unsatisfactory to some of his former students.

If variations such as these occur (and instances are coming to the writer's attention to suggest that they do), it is plain that absolute standardization of a technic is both difficult and highly undesirable. It *must* vary to accommodate the eyes of the different men who use it. It would help, however, if the different variations were known and recognized as modifications, each suited for its own purposes. To recognize them as such is the problem.

The most promising solution that has been suggested comes from one member of the Commission, R. W. French of the Army Medical Museum, at Washington, D. C. His suggestion is that authors of new technics be urged to send slides illustrating their procedure to some central bureau; and that others be secured to cooperate in securing similar slides to represent acceptable preparations by old procedures. The museum having charge of these slides will then agree to send them out to individuals wishing to check up their results with those of the author of the technic in use.

The services of the Army Medical Museum are offered in making this collection, and it is promised to announce the details of the plan at a later date. Meanwhile anyone interested is invited to communicate with R. W. French on the subject.

The plan seems practical in the case of those procedures that yield permanent preparations. It is obviously impossible in the case of material subject to disintegration or stains likely to fade. It also presupposes an experienced technician at the museum who would be able to judge as to whether a preparation many years old is still good enough to serve as a standard before sending it out to any correspondent. With these limitations the plan seems practical; at all events it is the only suggestion that has yet been made by means of which greater uniformity in staining procedures can be secured in the case of any complicated technic.

H. J. Conn.

THE CHEMICAL NATURE OF METACHROMASY

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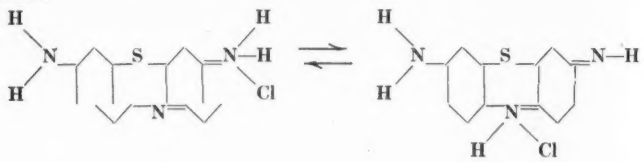
ABSTRACT.—**The phenomena of metachromasy are paralleled by analogous color changes which may be observed with aqueous solutions of dyes. It appears very probable that both types of phenomena arise from tautomerism in the structure of the dyes concerned. No adequate explanation of the behavior of dyes in these particulars has been proposed hitherto.**

A hypothesis is advanced which accounts for these phenomena in a satisfactory manner. It is held that the majority of dyes of aminated constitution are capable of undergoing a tautomerism which involves merely a redistribution of affinities within the dye molecule, resulting in a transition between structure of ammonium salt type and that of addition product type.

It was noted early in the development of staining that various basic dyes of the quinonimide and triphenylmethane classes are metachromatic. They act as true differential stains, dyeing certain tissue elements the color of the dyes themselves and other elements the color of the corresponding dye bases. According to Michaelis the development of the color of the dye bases (termed the metachromatic color of the stains) does not arise from alkalinity in the elements in question and does not involve any actual formation of the dye bases. The general character of the phenomenon under discussion renders it very probable that some type of tautomeric alteration in the dyes is involved. Employing thionin as a typical example of a metachromatic dye Michaelis[1] represents this tautomeric equilibrium in the following manner.



(I) (II)

He assumes that the staining of the cell nuclei (violet) is due to the presence of the dye therein in a structural form analogous to that represented by formula I and that the staining of mast cell granules (red) involves structural rearrangement to the form represented by formula II.

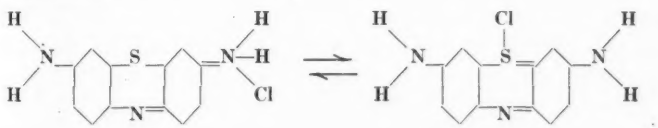
It should be noted that this hypothesis, in common with the alternate hypotheses, which will be referred to subsequently, carries no

[1]Michaelis, L. Einführung in die Farbstoffchemie für Histologen. Berlin, 1902.

implication respecting the actual nature of the dye retention. It is equally valid whether the staining is a physical phenomenon in which the dyes themselves, as represented in the formulae, are the agents, or a chemical phenomenon in which the staining agents are analogous compounds to the dyes in which the acid radicals of the dyes have been replaced by acid radicals present in the tissue

Although Michaelis' hypothesis appears to have met with wide acceptance among biologists it must be considered untenable. Evidence has been obtained that the basicity of the nitrogen atom which links the benzene nuclei in the thiazine structure is much weaker than that of the amino nitrogen atoms which are substituents of the benzene nuclei, and it seems very improbable, accordingly, that the dye form represented by formula II can actually exist. Even if the possibility of its existence is conceded, its formation would not, in the opinion of the writer, afford any adequate explanation of such a considerable alteration in color as is at issue. The argument on this point will be elaborated subsequently. It will be evident, finally, that this type of tautomerism is impossible in the case of triphenylmethane derivatives. The metachromatic phenomena observed with crystal violet are of an analogous type to those observed with thionin, and any acceptable hypothesis on the nature of metachromasy must account in a satisfactory manner for the behavior of both classes of dyes.

The equilibrium postulated by Michaelis may be revised to the following form:



(paraquinoid) (I) (orthoquinoid) (III)

and the attempt has been made to account for metachromasy on the basis of this tautomeric equilibrium between paraquinoid and orthoquinoid structure. Both dye forms have found advocates among competent dye chemists; and it is, perhaps, the present consensus of opinion that quinonimide dyes are essentially desmotropic, or in other words, capable of assuming either paraquinoid or orthoquinoid structure in accordance with varying conditions. Whereas, however, this revised equilibrium is consistent with accepted conceptions of chemical structure, it affords no more satisfactory explanation of metrachromasy than that proposed by Michaelis. It does not

account for radical alteration in color and is inapplicable to triphenylmethane derivatives. A new hypothesis is required which will offer a more adequate explanation of metachromatic phenomena and prove equally applicable with metachromatic dyes of all classes.

A digression is advisable here in order to point out that the phenomena of metachromatic staining are paralleled closely by corresponding alterations in the color of the aqueous solutions of many dyes with change in dye concentration.[2] The solution phenomena are exhibited by numerous dyes which do not afford effective metachromasy, but all metachromatic dyes are affected by variation in concentration and assume their metachromatic colors when their aqueous solutions are made sufficiently concentrated. Both thionin and crystal violet, for example, are red in concentrated aqueous solutions (and in the solid state) and become violet in aqueous solutions only at relatively considerable dilutions. There can be little question but that both the staining and the solution phenomena originate in a common cause and require a common explanation. In the subsequent discussion it will be convenient to consider the solution changes, primarily, but it should be understood that any adequate explanation of these will also afford a satisfactory explanation of metachromasy. Crystal violet will be considered by way of example, inasmuch as its behavior has defied explanation on the basis of all previous hypotheses of chemical tautomerism; but it will be understood that any conclusions derived from its consideration will have equal validity for many other dyes of aminated constitution including all metachromatic dyes.

In the solid state or in concentrated aqueous solutions crystal violet is red, with an absorption band which has its maximum at approximately 540 mμ. In dilute aqueous solutions and in both concentrated and dilute alcoholic solutions, it is violet, having a different band with maximum absorption at approximately 590 mμ. At intermediate concentrations in aqueous solution its color is intermediate between red and violet and both bands are present in varying degrees of ascendency. Analogous modifications of color and absorption with change in concentration in aqueous solution occur with the majority of dyes of aminated constitution, including all metachromatic dyes.

In the original discussion of these phenomena (see footnote 2) the possible operation of hydrolytic and electrolytic dissociation, solvation and molecular aggregation of solute was rejected and it was

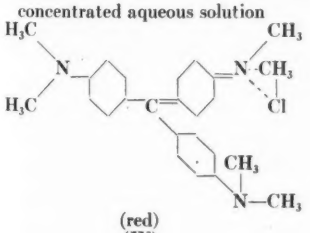
[2]Holmes, W. C. The Influence of Variation in Concentration on the Absorption Spectra of Dye Solutions. Ind. and Eng. Chem., 16, 35. 1924.

concluded that the phenomena probably constituted "evidence of a radical alteration within the dye molecule, involving a rearrangement in the distribution of affinity if not an actual atomic rearrangement." Subsequent investigations have strengthened the writer's belief that some type of molecular tautomerism is necessarily involved and a definite hypothesis is now advanced respecting the nature of the tautomerism in question.

Tautomerism may be defined broadly as molecular rearrangement brought about by change in conditions and resulting in the alteration of the properties of the substance in question. In the restricted sense it implies a definite migration of an atom or several atoms from one position in the molecule to another. This type of structural tautomerism is illustrated in the equilibria which have been written previously in this paper, in which a chlorine atom and a hydrogen atom are assumed to be labile, or capable of migration with change in conditions. It will be evident that this type of tautomerism cannot afford an explanation of the behavior of crystal violet. The only conceivable migration within the crystal violet molecule (see formula V), which would affect its color, would be that of the chlorine atom to the linking carbon atom; one which appears improbable from chemical considerations and which would, moreover, result in destruction rather than in mere modification of color, since it would result in the disruption of the quinoid structure. The hypothesis advanced herein postulates molecular rearrangement thru the redistribution of affinities within the dye molecule without any actual migration of labile atoms.

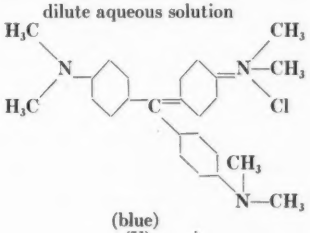
It is now suggested that dyes of aminated constitution commonly exist in the solid state and in concentrated aqueous solutions in the form of addition products. (The type of structure referred to will be made sufficiently clear in the subsequent discussion.) In dilute aqueous solutions and in both concentrated and dilute alcoholic solutions they exist as salts of the ammonium type. At intermediate concentrations in aqueous solutions both types of structure are found

concentrated aqueous solution



(red)
(IV)

dilute aqueous solution



(blue)
(V)

in tautomeric equilibrium. The same two types of structure account for metachromasy.

In the typical instance of crystal violet the tautomerism of the dye may be represented by the preceding formulae.

Formula V is the ammonium salt formula universally assigned to the dye and requires no comment. Formula IV has not been proposed hitherto so far as the writer is aware. It represents the dye as an addition product in which methyl chloride is bound to the amino nitrogen atom by *partial* valencies (or "residual affinity"), and in which the nitrogen atom in question is essentially trivalent rather than pentavalent. It is not a salt.

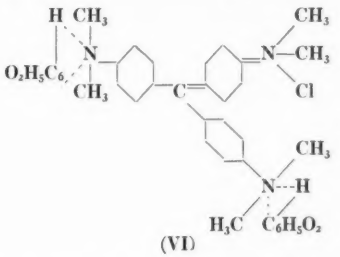
It is an essential feature of the hypothesis advanced that the change in valency of the amino nitrogen atom from three to five (or, in other words, the salt formation), which occurs in passing from formula IV to formula V is the *only* factor involved which would influence color. The formation of addition products of the type illustrated is without appreciable effect upon color (see below) and the color of the dye form represented by formula IV would not be influenced by the methyl chloride which is held by partial valencies. In other words the alteration in color which would result from the tautomerism represented would be neither more nor less than the alteration in color which results from salt formation at the amino group. With the type of equilibrium which was illustrated in discussing thionin, on the other hand, it will be noted that both tautomeric forms of the dye are true salts. Salt formation at the amino group is there accompanied by the disruption of salt structure elsewhere in the molecule. The precise effect of such tautomerism on color may only be conjectured, but it is certain that the opposed types of salt formation would exert effects upon color which would be similar in kind if not the same in degree and would tend to neutralize each other. Actual alteration in color might possibly result but the color change would certainly be much less than would result from the tautomerism herein proposed.

Under the hypothesis advanced, the observed color change in crystal violet is attributed to the conversion of the amino nitrogen atom from the trivalent to the pentavalent state. Altho the alteration in color is relatively extreme, being fully equivalent to the effect of all six methyl substituents in the amino groups, there can be no question but that the valency change (or salt formation) in question affords an adequate explanation. A second nitrogen atom in the crystal violet molecule may be converted from the trivalent to the pentavalent condition either by forming the diacid salt of the dye through the addition of mineral acids, or by converting the dye into

methyl green by the action of methyl chloride. In either case the result is essentially the same. The color is changed from violet to green and the absorption band shifted from 40 to 45 mμ toward the red end of the spectrum. The third amino nitrogen atom may also be converted into the pentavalent state by more drastic treatment along the same lines and both the triacid salt of the dye and octamethyl pararosanilin obtained. In either case, the result is the same. Both products are yellow and there cannot be the slightest question but that these are yellows of the "second order" and that here, again, a considerable deepening in color has resulted. Similar effects may be obtained with all triphenylmethane and quinonimide dyes, and the transition of their amino nitrogen atoms from the trivalent to the pentavalent condition is invariably accompanied by a very decided deepening in color. These color changes which are known to originate in salt formation at the amino groups are of the same general order of magnitude as the changes observed with variation in the concentration of aqueous solutions of aminated dyes and those observed in metachromasy.

The hypothesis advanced, accordingly, accounts in a thoroughly satisfactory manner for the color phenomena of crystal violet (and many other dyes) which appear to admit of no other possible adequate explanation.

Further justification for the assumption of the novel type of structure represented by formula IV has been found in the synthesis of a variety of products which the writer has recently prepared from basic dyes and cyclic derivatives containing phenolic groups.[3] It has been found, for example, that crystal violet and resorcin react with the formation of the product represented by the following formula



(VI)

This product is a crystalline compound of considerable stability which can be recrystallized repeatedly from water or alcohol without alteration. It contains two molecules of resorcin in combination with

[3]The investigation of these compounds will be detailed in an early publication.

one molecule of dye. Its color is identical with that of the dye itself and it undergoes the same decided modification in color with change in concentration in aqueous solution. It must necessarily be an addition product of the type represented, in which the resorcin molecules are bound to the unsaturated amino nuclei of the dye by partial valencies. It is broken down and the resorcin liberated, as would be anticipated, when mineral acids are added and the poly-acidic salts of the dye are formed.

Analogous compounds are formed by basic dyes with other substances of acid characteristics and some of the common forms in which dyes are marketed are of this type, altho their character does not appear to have been recognized. The "zinc chloride double salt" of methylene blue, for example, is an addition product in which one molecule of zinc chloride is bound to the unsaturated amino nuclei of two molecules of the dye by partial valencies. The oxalate of malachite green is a complex in which two molecules of the dye have entered into true salt formation with one molecule of oxalic acid and the remaining two unsaturated amino nuclei of the resulting dye salt have each added on a molecule of acid without entering into definite salt formation with it.

It is held that the demonstration of the occurrence of this addition type of structure in the complexes which are formed by basic dyes with substances of an acid character afford support for the assumption of the occurrence of the analogous type of structure in the dye itself, as represented in formula IV.

A NOTE ON THE STAINING OF THE SKELETON OF CLEARED SPECIMENS WITH ALIZARIN RED S

ALDEN B. DAWSON, *Department of Biology, New York University*

ABSTRACT.—**A selective, progressive method for staining the skeleton in cleared specimens, developed with rat material.**

Fix in 95% alcohol for at least 48 to 96 hrs. Even longer fixation is desirable. Then place in a 1% solution of KOH until the bones are clearly visible through the surrounding tissues. Transfer directly to a dilute solution of alizarin in KOH, one part alizarin to 10,000 parts of 1% KOH. Allow the stain to act until the desired intensity is attained. Fresh stain may be added if necessary.

Complete the clearing process, (1) in Mall's solution, water 79 parts, glycerine 20 parts and KOH 1 part; (2) in increased concentrations of glycerine. Store in pure glycerine.

The success of the method depends on obtaining the proper degree of clearing before staining. If the specimen is insufficiently cleared, a general staining of all tissues usually occurs.

The methods commonly used for obtaining a differential staining of developing bone in specimens already cleared or to be cleared (Lundvall 1905, Spalteholz, 1914; Batson, 1921), have involved the staining of the entire specimen in a rather concentrated solution of alizarin red S (sodium alizarin monosulfonate), followed by differential decolorization. The alizarin is ordinarily made up as an alcoholic solution, usually in 95 per cent. Such a solution stains the soft parts as well as the skeleton and must be followed by a decolorizing fluid. Decolorization is accomplished, either by using an acid alcohol, such as a one-half per cent solution of sulfuric acid in 95 per cent alcohol, or by allowing the specimen to stand in strong sunlight until the stain has been removed from the tissue surrounding the bones.

Decolorization by means of any acid solution is objectionable in a study of ossification on account of the danger of decalcifying minute ossification centers. Decolorization in strong sunlight while not open to the objection just raised for the acid method also has some drawbacks. In continuous cloudy weather it is quite obvious that no progress can be made and, in addition to this, many specimens prove very refractory to the sunlight method and the process of decolorization in these cases may take days, weeks, or even months.

In a study now in progress on the effect of sex on the rate of ossification in the fore and hind limbs of the albino rat, a modified method of staining with alizarin has been used which gives uniform and excel-

lent results. The modified method is one of progressive, selective staining rather than a method of differential decolorization, such as has been briefly referred to above. It has the advantage of introducing no factors of error due to possible decalcification and takes less time than bleaching in strong sunlight.

Legs of rats from one to thirty days of age have been prepared by this modified method. The animals are fixed *in toto* in 95 per cent alcohol for from 48 to 72 hours depending on the age of the animal. Prolonged fixation in the alcohol seems to render the tissue less liable to macerate in the potash solutions used later. The tissue is then placed in a one per cent solution of potassium hydroxide, Mall's (1902) modification of Schultze's (1897) method, for from 24 to 72 hours or until the bones are clearly visible through the muscle. The specimens are then placed in a dilute solution of alizarin and potash, one part alizarin to 10,000 parts 1 per cent potassium hydroxide. They are allowed to remain in this solution until the bones are stained the desired color. If the dye is absorbed from the solution before the maximum intensity is obtained, the specimen may be transferred to a fresh solution of stain. If clearing in the initial potash solution has been allowed to progress to the proper stage, nothing but the bone will take up the stain. If, however, the clearing has not been complete enough the muscles and other tissues take the stain almost as readily as the bone itself. Following the staining the tissues are placed in Mall's solution, water 79 parts, glycerine 20 parts and potash 1 part. When sufficiently cleared they are passed up thru increasing concentrations of glycerine and stored in pure glycerine.

I am indebted to Mr. Charles Spark for assistance in carrying on the experiments necessary to perfect this method of selective staining.

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AN IMPROVED STAIN FOR BACTERIA IN SOIL

H. J. Conn, *New York Agricultural Experiment Station, Geneva, N. Y.*

Abstract.—**A stain for bacteria in soil is proposed different from that originally employed in the introduction of pre-treatment of the soil smears with dilute acid. The procedure found best is: A suspension of soil in a 0.015% solution of gelatin is dried on a slide, treated 1–3 minutes in 40% acetic acid, washed and dried on a boiling water bath; while still on the water bath it is stained about one minute with 1% aqueous rose bengal. It is suggested however that under certain conditions better results may possibly be obtained by substituting hydrochloric for acetic acid and one of the phloxines or one of the erythrosins for rose bengal.**

In the last number of this journal the general properties of the fluorescein dyes as bacterial stains were discussed,[1] and it was shown that the majority of dyes in this group could be used for the purpose, as well as rose bengal which had been first used for staining bacteria in soil.[2] Rose bengal, however, was still considered best for this latter purpose, altho the phloxines and erythrosins were found to give good results. It was also pointed out that the behavior of the different dyes, when applied to soil suspensions, varied with the reaction of the material stained, and it was suggested that this might account for Winogradsky's better results with erythrosin.[3]

The idea thus suggested was followed up in the following way: Soil suspensions were prepared by the usual technic, and dried on microscopic slides. They were then immersed for a few minutes into some buffer solution having a known hydrogen-ion concentration. The reaction of the four buffer solutions used were approximately: pH = 4, pH = 6, pH = 8, and pH = 9.5. Thus treated the following results were obtained:

Rose bengal (C. I. No. 779) stained the bacteria in the soil quite well if the preparation had been treated at pH = 4, fairly well at pH = 6, but very poorly after treatment in either of the two alkaline solutions. In the latter case the trouble was that the dead organic matter was too deeply stained.

Erythrosin (either yellowish or bluish), on the other hand, was found to stain best following treatment at pH = 8 or 9.5, and very poorly following treatment at pH = 4. Phloxine B was found to

[1] Conn, H. J. and Holmes, W.C. Fluorescein dyes as bacterial stains. Stain Techn. 1, 87–95. 1926.

[2] Conn, H. J. The microscopic study of bacteria and fungi in soil. N. Y. Agr. Exp. Sta., Tech. Bul. 64. 1918.

[3] Winogradsky, S. Sur l'etude microscopique du sol. Acad. d. Sci., Com. Ren. 179, 367. 1924.

behave much like erythrosin; but it did not give such a good stain as the latter, the organic matter being in all cases too deeply colored.

Now the reaction of the soil used by Winogradsky when he compared rose bengal and erythrosin is not known to the author; but if it was a soil of more alkaline reaction than the one specially studied here, the reason why he found best results with erythrosin is readily explained by the above findings.

A further possible explanation of the difference between the author's results and those of Winogradsky may readily occur to anyone who has compared the two methods. The original technic called for a 0.015% solution of gelatin as a fixative; while Winogradsky's modification requires a 0.1% (or for certain purposes a 1%) solution of agar. An agar fixative preceding erythrosin is also used by Thornton and Gangulee[4] in their more recent modification. The probable reason for choosing an agar fixative is that the weak solution of gelatin originally employed does not have sufficient adhesive power for sandy soils; agar may be used in higher concentration but gelatin cannot, because in that case the whole background stains pink. It seemed possible, upon studying Winogradsky's paper, that erythrosin may give better results in the presence of agar than rose bengal.

Investigation shows that this is not the case, however. Employing the soil most frequently studied by the author, the presence of agar instead of gelatin does not seem to have any effect on the staining qualities of the two dyes. This makes the conclusion inescapable that the better results obtained by Winogradsky with erythrosin are due either to differences in the soil or to mislabeling of the dyes on the market. Mislabeling of dyes in this group proves to be very common.

The improved behavior of rose bengal following pre-treatment with an acid solution was so manifest as to suggest the possibility that the action of the phenol in the formula originally proposed for this technic might be merely to control the reaction. It is well known that acid dyes act more strongly if the reaction of the dye-bath is acid than if it is neutral; (see Briggs and Bull[5]). Accordingly the experiment was repeated in part, using a plain aqueous (one per cent) solution of the dye; a good stain was obtained following treatment at pH = 4.

These results tended to confirm the above mentioned inference and suggested that even better results might be obtained with a higher acid. Briggs and Bull found greater dye absorption, in the case of three different acid dyes of the azo group, to occur at a reaction near

[4]Thornton & Gangulee.

[5]Briggs, T. R., and Bull, A. W. The physical chemistry of dyeing. Acid and basic dyes. J. Phys. Chem., **26**, 845–875. 1922.

pH = 2 than in solutions nearer neutrality. To investigate the matter two different acids were used: a 40% solution of acetic acid; and a solution of hydrochloric acid about tenth normal. Both of these acids were too strong to mix with the dye without precipitating the color acid; so the plan of pre-treatment was continued.

Using 40% acetic acid for treating the soil smears before staining, the results were unexpectedly fine. The bacteria stood out more sharply than in any preparation ever before examined. The same soil that had been used thruout this study showed so many more bacteria than by any previous technic that, until the work had been repeated, it was thought that the preparations must have been contaminated with bacteria from some outside source. Using the dilute hydrochloric acid, the results were not so good; there was too much tendency for the dead organic matter to take the stain and in that way to obscure the bacteria.

After obtaining these splendid preparations, the technic was repeated with other dyes of the same group. It was found that erythrosin Y (C. I. No. 772) and phloxine B (C. I. No. 778) sometimes gave results almost as good as rose bengal. Erythrosin B (C. I. No. 773) and phloxine (C. I. No. 774) gave fairly good results. Ordinarily the results following acetic acid were better than after hydrochloric; but in the case of phloxine (No. 774) the reverse seemed to be the case. It is probably possible in studying some particular soil to obtain good results with almost any of these five dyes provided the proper form of pre-treatment with acid is employed. Nearly all the other dyes described in the earlier paper[6] were tried, but did not give good preparations.

It was a matter of some interest that the best results following acetic acid were obtained with the bluer phloxine but with the yellower erythrosin. Now erythrosin Y is more weakly acid than erythrosin B, and phloxine B more weakly acid than phloxine. This suggests that the behavior of these dyes following treatment with acetic acid depends more upon the strength of the dye acid than upon the shade of the dye. If this same relation should hold true in the case of rose bengal, the bluer rose bengal (C. I. No. 779) should give a better stain than the yellower homolog (C. I. No. 777); but as the former is the only one of the two so far obtained, this comparison could not be made.

The theory involved is an interesting matter for speculation; but the data at hand are insufficient to warrant any conclusions. The improved staining after treatment with acid may be due to greater

[6]See footnote 1.

adsorption of the dye at an acid reaction; or it may result from the precipitation within the bacterial cell of the color acid by means of the stronger acid retained after the pre-treatment. Regardless of the theory, it is certain that the new procedure is a great improvement over any previously recommended. The technic is as follows:

Make a suspension of soil in nine times its weight of an 0.015% solution of gelatin. Smear a drop of this in a thin film on a slide (both the amount used and the size of the smear must be measured if the number of bacteria is to be counted). Immerse for from one to three minutes in a 40% solution of acetic acid or a 10th normal solution of hydrochloric acid. Wash off the excess acid briefly, and dry on a flat surface over a boiling water bath. While still on the water bath cover the film with a 1% aqueous solution of rose bengal, phloxine B, or erythrosin Y, and allow it to stain for about one minute. The combination giving best results in the present investigation is rose bengal following acetic acid; but in other laboratories, or with the use of other soils, different combinations of the above named acids and dyes may prove more satisfactory.

BRILLIANT GREEN BILE MEDIA[1]

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ABSTRACT.—**An attempt is made to determine the optimum concentration of bile and brilliant green in brilliant green lactose peptone bile medium, for use in the presumptive test in the bacteriological examination of water. Comparisons, showing the effect of bile and brilliant green upon the development of the colon organism, were made by making actual bacterial counts after a definite short incubation period. The results indicate that the original medium described by Muer and Harris, (5% dried bile, 1% peptone, 1% lactose, and 1:10,000 billiant green) when adjusted to pH = 7.1, did not show an appreciable inhibition of the colon organism. It is further shown that a medium containing 2% bile, 1% peptone, 1% lactose and 1:75,000 brilliant green supports a more rapid development of the colon organism at pH = 6.9 than does the original brilliant green bile medium at its optimum pH.**

During the past few years an increasing interest has been shown in the brilliant green bile medium originally described by Muer and Harris.[2] This medium was devised to stimulate the growth of the colon organism,[3] whose presence is indicative of contamination, and to eliminate other gas-forming bacteria technically known as "non-confirming gas producers." Considerable criticism has been current in recent years to the effect that the brilliant green might possibly inhibit the colon organism as well as the other gas-forming bacteria.

It was the purpose of one section of Committee No. 1 on Standard Methods of Water Analysis, of the American Water Works Association, to suggest a medium containing 1% lactose, 1% peptone, and the combination of bile and brilliant green that would show maximum development of the colon organism and a minimum development of the non-confirming lactose-splitting aerobic spore formers.

This problem was subdivided into three sections; namely,

[1]This report, which was read at the 46th Annual Convention of the American Water Works Association held at Buffalo, N. Y., June 7, 1926, represents the work performed in the Bacteriological Laboratory of the Digestive Ferments Company on an assignment of Committee No. 1, Standard Methods of Water Analysis.

[2]Muer, T. C. and Harris, R. L. The value of brilliant-green in eliminating errors due to the anaerobes in the presumptive test for *B. coli*. Am. J. Pub. Health, **10**, 874, 1920.

[3]To avoid taking sides in a controversial question of nomenclature, the term "colon organism" is here used instead of a specific name. Wherever this term is employed it will be understood to refer to the organism producing acid and gas from glucose, lactose and fructose, but not from sucrose, which is variously denoted in the literature as *Aerobacter coli*, *Bacillus coli*, *Bacterium coli*, and *Escherichia coli*.

1. The determination of the optimum concentration of bile for the maximum rate of growth of the colon organism.

2. The determination of the optimum concentration of brilliant green to use with the bile as determined in problem 1.

3. To determine the optimum H-ion concentration to be used with the combination selected in problems 1 and 2.

The method used to determine these factors was the measurement of a comparative increase in numbers of organisms, after a definite short incubation period, in a medium varying only one factor at a time, and using suitable controls. The technic used is given in detail:

Both freshly isolated and old laboratory strains of the colon organism were utilized in these studies. To obtain actively growing cultures, transfers were made daily for 5 to 7 days in Dunham's peptone solution (1% peptone and 0.5% sodium chloride), using a standard loop. The inoculum was prepared by pipetting 1 cc. of each of the cultures into a 99 cc. dilution blank, and making additional dilutions so that each cubic centimeter of the media contained from 60 to 100 cells at the beginning of the experiment. Usually three different strains of the colon organism were mixed and used at the same time. This tended to eliminate any individual effects of any one strain.

The media to be tested were distributed in 10 cc. lots in tubes of uniform bore. After the tubes had reached a constant temperature in a 37°C. water bath, they were seeded, generally, with a 1:1,000,000 dilution of the cultures used, at one minute intervals. Following thoro mixing, the tubes were incubated for 5 or 6 hours, keeping the water bath at 37°C.

To determine the number of organisms seeded, the inoculum was always plated on nutrient agar.

After the expiration of the 5 or 6 hour incubation period, 1 cc. of each medium was removed from the tubes at one minute intervals, and suitable dilutions made so that nutrient agar plates from each tube might be obtained containing between 30 and 300 colonies. Duplicate plates were always poured and counts reported as an average of the two plates.

All plates were incubated at 37°C. for 48 hours and bacterial counts made at the end of this period.

In order to facilitate the comparison of results, the counts of the controls were given the value of 100 as unity and other counts calculated proportionately.

PART I

THE OPTIMUM CONCENTRATION OF BILE

A basic medium containing 1% lactose and 1% peptone was used as a control. Other media containing 1, 2, 3, 4, and 5% bile were prepared by the addition of dehydrated bile to this basic medium. The reaction of all media was between pH = 6.8 and 6.9. The variable factor then, in this series of media, was the concentration of bile. It was considered reasonable to suppose that a variation in numbers of organisms, after a definite short incubation period, would be dependent upon the effect of the bile on the organism.

Table 1 gives the results of six series of tests with the control medium reduced, for the sake of an easy comparison, to a unity of 100. Three different lots of bile and several combinations of five strains of the colon organism were used in securing this data.

TABLE 1. EFFECT OF BILE IN VARYING CONCENTRATIONS UPON RATE OF GROWTH OF THE COLON ORGANISM.

Conc. of Bile (dry)	Relative Bacterial Counts						
	1	2	3	4	5	6	Average
None	100*	100	100	100	100	100	100
1%	517	126	110	141	208	219	220
2%	668	143	170	165	290	211	275
3%	450	129	121	157	218	241	219
4%	275	81	88	157	192	35	121
5%	150	75	68	145	180	44	110

*To facilitate interpretation, the controls were given the value of 100 as unity and other counts calculated proportionately.

It is quite apparent from this table that the 1, 2, and 3% concentration of bile exerted a distinct stimulative effect in every case. The 2% appeared to be almost consistently higher than the other concentrations, and was selected as being a more desirable concentration to use.

To substantiate the results obtained in Table 1, additional tests were made using the control, 2% and 5% bile media only. The results of these tests are given in the following table.

TABLE 1A. EFFECT OF BILE IN VARYING CONCENTRATIONS UPON RATE OF GROWTH OF THE COLON ORGANISM.

Conc. of Bile (dry)	Relative Bacterial Counts									
	1	2	3	4	5	6	7	8	9	Average
None	100*	100	100	100	100	100	100	100	100	100
2%	825	888	575	635	302	520	263	400	480	543
5%	300	316	195	198	191	—	—	—	—	240

*To facilitate interpretation the controls were given the value of 100 as unity and other counts calculated proportionately.

The data given in Tables 1 and 1A indicate that 2% bile always stimulated the development of the colon organism and that 5%

showed stimulation, with a few exceptions, but in a lesser degree than did the 2% bile.

Part II

Effect of Various Concentrations of Brilliant Green with 2 or 5% Bile on the Development of the Colon Organism

A. With 2% Bile

Employing the technic as given above, the effect of brilliant green was determined by the addition of varying concentrations of the dye to the basic medium containing 2% bile and having a pH of 6.8.

This basic medium with 2% bile, without the dye, served as a control. The variant in this series was the concentration of the dye.

Table 2. Effect of Brilliant Green in Varying Concentrations on Rate of Growth of the Colon Organism in Lactose Peptone Bile Containing 2 % Bile (Dry).

Conc. of Br. Green	Relative Bacterial Counts					
	1	2	3	4	5	Average
None	100*	100	100	100	100	100
1–15,000	50	56	49	66	43	53
1–20,000	56	70	64	74	55	64
1–50,000	140	88	86	104	87	101
1–75,000	105	120	77	132	95	105
1–100,000	94	103	86	121	100	101

*To facilitate interpretation the controls were given the value of 100 as unity, and other counts calculated proportionately.

From the figures, as given in Table 2 it is quite apparent that with a 2% bile medium a concentration of 1:75,000 brilliant green did not inhibit the development of the strains of the colon organism used, and may be employed without the danger of causing an appreciable inhibition.

B. With 5% Bile

Using the same method as in previous work, the following data were obtained. The hydrogen ion concentration, was pH = 6.9 for all media.

Table 3. Effect of Brilliant Green in Varying Concentrations on Rate of Growth of the Colon Organism in Lactose Peptone Bile Containing 5% Bile (Dry).

Conc. of Br. Green	Relative Bacterial Counts					
	1	2	3	4	5	Average
None	100*	100	100	100	100	100
1–5,000	—	—	—	—	25	25
1–10,000	124	104	117	98	149	118
1–15,000	142	100	100	110	111	112
1–20,000	131	111	126	123	118	122
1–25,000	154	91	113	113	139	121
1–30,000	107	121	88	116	115	109

*To facilitate interpretation the controls were given the value of 100 as unity, and other counts calculated proportionately.

The single test using 1:5000 concentration of brilliant green showed a decided inhibition on the development of the colon organism by this concentration of dye. Concentrations of brilliant green from 1:10,000 to 1:25,000 showed about the same slightly stimulatory effect. The purpose of this medium is to inhibit the non-confirming lactose splitting organisms, and at the same time show no inhibition of the colon organism. Since a 1 to 10,000 concentration of brilliant green did not markedly inhibit the growth of the colon organism, and since this amount of dye has given practical results in inhibiting non-confirming lactose splitting organisms, it is recommended that a 1:10,000 concentration of brilliant green be used with 5% bile as was originally suggested by Muer and Harris.

Part III

Effect of H-ion Concentration on the Development of the Colon Organism in Brilliant Green Bile Media

A. With 2% Bile and 1:75,000 Brilliant Green

A culture medium containing 1% lactose, 1% peptone, 2% dehydrated bile and 1:75,000 brilliant green was prepared. This was divided into four parts and each adjusted to a different reaction as shown in Table 4. The effect of the different reactions within media of the same composition was determined according to the technic used in the previous studies.

Table 4. Effect of H-ion Concentration on the Development of the Colon Organism in a Medium Containing 2% Bile and 1:75,000 Brilliant Green.

pH	Bacterial Counts			
	1	2	3	4
6.2	100,000	350,000	200,000	200,000
6.9	2,000,000	2,000,000	1,300,000	900,000
7.4	1,500,000	1,200,000	1,600,000	500,000
7.8	350,000	700,000	400,000	300,000

The data given in Table 4 show that, with these organisms, there is a distinct inhibition at pH = 6.2 and a lesser inhibition at pH = 7.8. With 2% bile and 1:75,000 brilliant green maximum growth was obtained at pH = 6.9.

B. With 5% Bile and 1:10,000 Brilliant Green

From the data given in Table 3, using a 5% bile medium at pH = 6.9 the most suitable concentration of brilliant green was 1:10,000. To determine the optimum H-ion concentration with 5% bile and 1:10,000 brilliant green, media containing 1% lactose, 1% peptone, 5% bile and 1:10,000 brilliant green were adjusted to various pH-values as indicated in Table 5. These media were seeded and incubated as in previous work and the bacterial counts are given in the following table.

TABLE 5. EFFECT OF H-ION CONCENTRATION ON THE DEVELOPMENT OF THE COLON ORGANISM IN A MEDIUM CONTAINING 5% BILE AND 1:10,000 BRILLIANT GREEN.

pH	Bacterial Counts			
	1	2	3	4
6.1	110,000	200,000	200,000	200,000
6.5	450,000	460,000	900,000	900,000
7.1	1,500,000	1,700,000	1,600,000	1,500,000
7.3	2,000,000	1,100,000	1,400,000	1,300,000
7.6	1,200,000	1,400,000	800,000	800,000

Comparing the bacterial counts given in the table, a medium containing 1% lactose, 1% peptone, 5% bile and 1:10,000 brilliant green with an initial H-ion concentration between pH = 7.1 and 7.3 was the optimum for the development of the colon organism.

CONCLUSIONS

Concentrations of bile from 1 to 3% always stimulated the development of the colon organism. Concentrations of 4 and 5% bile gave variable results.

Greatest stimulation was evident in media containing 2% bile.

With 2% bile a concentration of 1:75,000 brilliant green was the largest amount of this dye that could be used without producing inhibition of the colon organism.

With 5% bile a concentration of 1:10,000 brilliant green was the largest amount of this dye that could be used without producing inhibition of the colon organism.

In 2% bile media containing 1:75,000 brilliant green the optimum reaction for the growth of the colon organism was at pH = 6.9.

In 5% bile medium containing 1:10,000 brilliant green the optimum reaction for the growth of the colon organism was at pH = 7.1.

In these studies a medium containing 1% lactose, 1% peptone, 2% bile and 1:75,000 brilliant green with an initial reaction of pH = 6.9 gave optimum conditions for the development of the colon organism.

It is imperative that these media be prepared in such a manner that after the water sample has been added the ingredients be present in the proper concentration.

A CONSIDERATION OF FRENCH'S TEST OF BASIC FUCHSIN FOR ENDO'S MEDIUM

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SUMMARY.—**In describing a method of testing for the return of color in decolorized fuchsin for use in Endo Medium, French states that variations in hydrogen ion concentration fail to influence the appearance of color in this medium.**

Duplications of this test were made using alcoholic and aqueous solutions of fuchsin and both sodium sulfite and sodium bisulfite as decolorizing agents.

In the decolorized alcoholic solutions of fuchsin the color failed to reappear when formalin was added, but a small amount of a weak solution of lactic acid caused the color to return.

Alcoholic solutions of fuchsin failed to decolorize in sodium bisulfite solutions until a few drops of NaOH were added. The color, then, reappeared immediately.

Solutions of peptones to which fuchsin had been added were substituted for the original fuchsin solution. Alcoholic and aqueous solutions of fuchsin were added to equal amounts of a 1% peptone solution. The peptone solutions varied in their hydrogen ion concentration and the results showed that those which were neutral decolorized readily while the more acid solutions were but partially decolorized.

Fuchsin decolorized according to results found in this test, was not satisfactory in the Endo medium, especially in the case of the aqueous solutions of fuchsin.

Experiments which were carried on by other workers and checked with this method all indicated that some acid is necessary to secure the restoration of color.

In a recent article R. W. French[1] states that the basic fuchsin plus sodium sulfite in the Endo Medium acts largely as a modification of Schiff's reagent for detecting aldehydes, and is practically independent of the hydrogen-ion concentration. In fact he definitely says it may be proven experimentally, that, "sensible variations in hydrogen-ion concentration utterly fail to influence the appearance of color in such a medium." "With this fact established," says French, "the following test for the suitability of a basic fuchsin for use in Endo medium is suggested. In a test tube take 10 cc. of a 0.5% aqueous solution of basic fuchsin. Determine the amount of 0.5% aqueous sodium sulfite

[1]French, R. W. Basic Fuchsin for Endo Medium. Jour. Lab. and Clin. Med. 11, 571. 1926.

solution necessary to effect decolorization. Then determine the amount of 0.5% formalin (40% formaldehyde) necessary to restore a distinct fuchsin color."

The first statement in regard to the hydrogen-ion concentration having no effect on the restoration of color seemed open to question in view of certain results obtained by the writer in a series of experiments carried on with Endo's medium.[2] A check on French's method and its application was therefore undertaken.

The experiment was planned as follows: (1) To duplicate the test described by French, using at least two different fuchsins. (2) To duplicate the test, substituting alcoholic solutions for the aqueous. (3) To use alcoholic and aqueous solutions of fuchsin in connection with the various peptones.

Robinson and Rettger[3] have advocated the use of sodium bisulfite as a decolorizing agent in this medium as a substitute for sodium sulfite. Sodium bisulfite, or acid sulfite, is very highly acid and experiments carried on by the writer, intended to duplicate the work of Robinson and Rettger have not been successful, since the resulting medium was so acid that the colon organisms which were inoculated on it did not grow. Even when this medium was adjusted to neutrality, the organisms refused to grow. In order to test this method further, it was decided to duplicate the experiments outlined above, using sodium bisulfite instead of sodium sulfite as a decolorizing agent. The results of this test were interesting.

The basic fuchsin was made up into 0.05% aqueous and 0.05% alcoholic solutions. Ten cc. of each of these solutions were placed in clean test tubes, and a 0.5% aqueous solution of sodium sulfite was added drop by drop until the fuchsin solution was decolorized. The amount of sodium sulfite was then recorded and a 0.5% solution of formalin (40% formaldehyde) was added drop by drop until the original color was restored. There were two brands of fuchsin available for this work, one a National Aniline Chemical Company product and the other a Coleman Bell product, uncertified. Both of these fuchsins have given good results in Endo's medium. This experiment was repeated using a 0.5% solution of sodium bisulfite in place of the sodium sulfite solution for decolorizing.

The hydrogen-ion concentration of sodium sulfite solutions was taken colorimetrically. The 0.5% aqueous solution of sodium sulfite

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[2]Genung, E. F. Endo Agar as Affected by Petptone. Science, **59**, 282. 1924.
[3]Robinson, H. C. and Rettger, L. F. Studies on the use of Brilliant Green and a Modified Endo's Medium in the Isolation of Bacillus typhosus from Feces. Jour. Med. Res., New Series. **30**, 363, 1916.

had a pH of 9.8 and that of the sodium bisulfite solution was pH = 4.4. The results of this test are recorded in Table 1.

TABLE 1. RESULTS OF FRENCH'S TEST WITH AQUEOUS AND ALCOHOLIC SOLUTIONS OF FUCHSINS DECOLORIZED WITH SODIUM SULFITE AND SODIUM BISULFITE.

Fuchsin	Amt. sodium sulfite to decolorize	Amt. 0.5% formalin to restore color	Amt. sodium bisulfite to decolorize	Amt. 0.5% formalin to restore color
C. B. Aqu.	.01 c.c.	.08 c.c.	.02 c.c.	.16 c.c.
N. A. C. Aqu.	.02 c.c.	.06 c.c.	.03 c.c.	.10 c.c.
C. B. Alc.	.74 c.c.	1.0 c.c. (No change)	No decolorization when added in excess.	
N. A. C. Alc.	.67 c.c.	1.0 c. c. (No change)	No decolorization when added in excess.	

The color restored in the aqueous solutions of the fuchsin was not so deep as in the original solution but doubling and trebling the amount of formalin did not change the intensity of color after the maximum color depth was reached.

When the alcoholic solutions of the fuchsins were decolorized by the addition of the 0.5% sodium sulfite solution and the 0.5% solution of formalin was added, no color returned, even after 1 c.c. of the formalin solution had been added. The addition of 0.03 c.c. of a 5% solution of lactic acid restored the decolorized fuchsin to the original color. A check on this test was made by adding 0.03 c.c. of a 5% solution of lactic acid to the decolorized fuchsin before adding the 0.5% solution of formalin. The latter was added drop by drop; 0.06 c.c. was needed to restore the color to its original depth in the case of the N. A. C. product, and 0.07 c.c. was used in the C. B. fuchsin.

The alcoholic solutions of the fuchsins resisted decolorization by sodium bisulfite even when used in as high amounts as 10 c.c. of the latter solution. If a drop of 10% NaOH was added to the solutions they decolorized immediately. To test this further, 10 c.c. of each of the 0.05% alcoholic solutions of the fuchsins was taken and 1 c.c. of the sodium bisulfite solution added to each, after which they were decolorized by adding a 5% solution of NaOH in 0.01 c.c. amounts until the fuchsin solutions were completely decolorized. It took 0.05 c.c. of the 5% NaOH solution to decolorize the C. B. fuchsin and 0.03 c.c. for the N. A. C. fuchsin.

To another 10 c.c. of each of the alcoholic fuchsin solutions the same amounts of 5% NaOH was added (i.e., 0.05 c.c. to the C.B. and 0.03 c.c. to the N. A. C. fuchsin), and no sodium bisulfite was added. After standing from five to ten minutes, the color had completely disappeared. To these decolorized tubes was added 0.01 c.c. of 5% lactic acid which restored the color to its original intensity.

An alcoholic solution of one of the fuchsins was placed in 10 c.c. portions in each of two test tubes and to each portion was added 0.05 c.c. of a 5% solution of NaOH. The contents of both tubes decolorized on standing about ten minutes. To one of these tubes was added 0.025 c.c. of a 5% solution of lactic acid and 0.025 c.c. of a 0.5% solution of formalin. The color was completely restored. To the second tube was added 0.05 c.c. of a 5% solution of lactic acid. The color returned but not to the intensity of the original solution, the subsequent addition of 0.005 c.c. of 0.5% solution of formalin restored the color completely.

Finely graduated serological pipettes were used for this work and the results were carefully checked over to avoid errors, especially when minute amounts were used. Five per cent solutions of sulfuric and hydrochloric acids were substituted for lactic acid and the results were the same.

TABLE 2. RESULTS OF FRENCH'S TEST WITH AQUEOUS AND ALCOHOLIC SOLUTIONS OF FUCHSINS DECOLORIZED WITH SODIUM SULFITE AND SODIUM BISULFITE AND ADDED TO PEPTONE SOLUTIONS WITH REACTION UNADJUSTED.

Peptone solution	Ph.	Aq. fuchsin sulfite solution	Color shade No.	Aq. fuchsin bisulfite solution	Color shade No.	Alc. fuchsin sulfite solution	Color shade No.	Alc. fuchsin bisulfite solution	Color shade No.
Difco	7.0	Trace pink	2.0	Trace pink	1.0	Trace pink	1.5	Deep pink	8.0
Witte	7.0	Faint pink	3.0	Trace pink	1.5	Faint pink	2.0	Deep pink	7.5
Park Davis	6.4	Rose pink	6.0	Rose pink	6.0	Pink	4.0	Red	9.0
Armour	6.2	Rose pink	6.5	Faint pink	3.0	Deep pink	5.5	Red	9.0
Fair-child	5.4	Rose pink	7.0	Rose pink	6.0	Deep pink	8.0	Red	1.0

The third part of the plan was to use peptone solutions in connection with the fuchsins, since it had been found in some previous experiments that peptones seem to have some effect on the restoration of color in Endo plates. Accordingly 1% solutions of 5 different brands of peptones were prepared. The hydrogen-ion concentration of each solution was determined, colorimetrically. To 5 c.c. of each peptone solution was added 5 c.c. of a decolorized aqueous solution of basic fuchsin. This fuchsin was decolorized on the basis of the results recorded above. One set of tubes was decolorized with a 0.5% solution of sodium sulfite, the other with a 0.5% solution of sodium bisulfite. The test was also duplicated using alcoholic solutions of fuchsin instead of aqueous solutions.

When the aqueous solution of fuchsin was decolorized with the sodium bisulfite solution and added to the peptone solutions, the

differences in the amount of color which appeared was about the same as that which appeared in the tubes decolorized with sodium sulfite, except the shade of color was in all cases less deep. If we denote the color shades by numbers, using No. 1 to indicate a very faint trace of pink and No. 10 to denote a deep rose red, the shades of color in the various tubes would range as recorded in Table 2. It will be noted that the peptone solutions vary considerably in their hydrogen-ion concentrations and that the color deepens in intensity in the more acid solutions.

The peptone solutions were then adjusted to pH = 7.0, and to 5 c.c. of each was added 5 c.c. of aqueous basic fuchsin decolorized with sodium sulfite and to another set 5 c.c. of aqueous basic fuchsin decolorized with sodium bisulfite. Duplicate tubes were prepared of

TABLE 3. RESULTS OF FRENCH'S TEST WITH AQUEOUS AND ALCOHOLIC SOLUTIONS OF FUCHSINS DECOLORIZED WITH SODIUM SULFITE AND SODIUM BISULFITE AND ADDED TO PEPTONE SOLUTIONS WITH THE REACTION ADJUSTED TO pH = 7.0.

Peptone solution	pH	Aq. fuchsin sulfite solution	Color shade No.	Aq. fuchsin bisulfite solution	Color shade No.	Alc. fuchsin sulfite solution	Color shade No.	Alc. fuchsin bisulfite solution	Color shade No.
Difco	7.0	Faint pink	2.0	Trace pink	0.5	Faint pink	2	Pale pink	3.0
Witte	7.0	Faint pink	2.0	Trace pink	0.5	Faint pink	2	Faint pink	2.0
Park Davis	7.0	Faint pink	2.5	Trace pink	0.5	Faint pink	2	Pink	3.5
Armour	7.0	Faint pink	2.5	Trace pink	0.5	Faint pink	2	Pink	3.0
Fair-child	7.0	Faint pink	2.5	Trace pink	0.5	Faint pink	2	Pink	3.0

the alcoholic fuchsin. The results are recorded in Table 3. It will be seen by this table that the aqueous and alcoholic solutions of the fuchsin when decolorized by sodium sulfite varied but little in the color which returned, all being a faint pink color while the color was much fainter in the aqueous solutions of fuchsin decolorized with sodium bisulfite than in the alcoholic solutions which were decolorized by the same reagent.

Endo agar was prepared using aqueous fuchsin decolorized according to the test just described. The results were very unsatisfactory. The growth was scant and no color appeared in the colonies of either the colon or the aerogenes types. All attempts to obtain a satisfactory reaction with a decolorized aqueous fuchsin in Endo's medium failed; but with alcoholic fuchsin the results were successful if the fuchsin was used in the proper dilutions.

In connection with the work of DeBord[4] (1917), some tests had

[4]DeBord, G. G. The Fuchsin-Aldehyde Reaction on the Endo Medium. Jour. Bact., **2**, 309–314. 1917.

been made previous to the experiments already described. These tests form an interesting comparison with French's test. DeBord, testing for the kind of reaction which causes the restoration of the fuchsin color in the colon colonies on Endo agar, tried the effect of various acids and formalin on this agar.

He made saturated aqueous solutions of basic and acid fuchsins which he decolorized with sodium sulfite in one instance and sodium bisulfite in another. He tried the effect of weak solutions of different acids, of the acid solutions plus formalin, and of formalin alone on the restoration of color in the decolorized fuchsin solutions. He summarizes his results, briefly as follows: "With basic fuchsin the mineral acids produced a straw-colored solution (when added to a colorless fuchsin-sulfite solution). The organic acids showed a slight reaction which might be produced by traces of formaldehyde as an impurity, and perhaps, also, by the formation of a strong base and weak acid combination. The reaction with formaldehyde alone was negative, but with an acid also a very strong reaction was obtained. This showed that the coloration with formaldehyde is dependent upon an acid medium."

DeBord also poured Endo plates made from basic fuchsin decolorized with sodium sulfite. On these plates he placed loopfuls of various organic and inorganic acids and formalin, then he allowed them to incubate for 24 hours at 37°C. He used N /25 N /50 and N /100 solutions of the acids and 40% formaldehyde. He summarizes his results as follows: "The basic fuchsin shows a coloration with concentrated organic acids, but none with concentrated mineral acids. *Formaldehyde alone does not give a reaction*, but when an acid is present a pronounced red color is produced."

He also tested acid fuchsin but found that it did not differentiate sharply between aldehydes and acids. When he used sodium bisulfite as a decolorizing agent, his results were as follows, "The organic acids did not produce quite so strong a color as the mineral acids. The basic fuchsin did not give a permanent red color with either mineral or organic acids. Formaldehyde gave a strong coloration without the addition of acid, because the bisulfite contains an acid radical which will serve to carry out the reaction. The results upon Endo plates were the same as with fuchsin reduced with the sodium sulfite solution."

DeBord's tests were checked over by the writer with aqueous solutions of basic fuchsin in test tubes. He does not state whether he used aqueous or alcoholic solutions for his Endo plates. Since the original method of making Endo agar and nearly all of the standard

modifications specify the use of a saturated alcoholic solution of basic fuchsin, the work of DeBord was repeated with alcoholic solutions of basic fuchsin instead of aqueous solutions.

Plates were poured from Endo agar made up with an alcoholic solution of basic fuchsin decolorized with sodium sulfite, the reaction of the agar having previously been adjusted to pH = 7.4. After the agar had become hard, the following test was made: Four drops of different dilutions of an acid were placed some distance apart on a plate. One drop of formalin (40% formaldehyde) was added to each of two of these drops. On one plate drops of formalin alone were used. These plates were allowed to stand and in a short time definite results could be recorded. These are shown in Table 4.

TABLE 4. RESULTS OBTAINED BY DROPPING ACID AND FORMALIN SOLUTIONS ON PLATES OF ENDO AGAR.

Kind of Acid	Acid alone	Acid plus formalin
5% Lactic	—	++++
10% Lactic	++	++++
5% Acetic	++	++++
2% Acetic	—	++++
5% Sulfuric	++	++++
.02% Sulfuric	—	++++
3% Hydrochloric	—	++++
N/20 Hydrochloric	—	++++

Formalin alone formed a deep fuchsin red.

Endo agar, made up by decolorizing the fuchsin with sodium bisulfite according to the method given by Robinson and Rettger, was poured into plates. The agar was adjusted to a pH = 6.8. Neither mineral nor organic acids restored the color on these plates, but acid and formalin produced a deep red color and formalin alone also produced a deep red. This corresponded with the results on the plates in which the fuchsin was decolorized with sodium sulfite, but does not correspond in either instance with the results obtained by DeBord.

A series of tubes were prepared as follows: Ten c.c. of a 2.5% solution of sodium sulfite was placed in each of ten tubes. To each tube was added 0.5 c.c. of an alcoholic solution of basic fuchsin which had been diluted to the proper strength for the Endo medium. This was completely decolorized. Into another set of ten tubes was placed 5 c.c. of a 10% solution of sodium bisulfite and 0.5 c.c. of an alcoholic solution of basic fuchsin. This was also completely decolorized. To each of the first four tubes in both series was added 5 drops of a 5% solution of the following acids respectively: lactic, acetic, sulfuric, and hydrochloric. To the next four tubes was added 5 drops of the same acids together with 5 drops of formalin and to the ninth tube formalin alone was added. The tenth tube was kept for a control. The results of this test are shown in Table 5.

There was a stronger reaction with the sodium bisulfite than with the sodium sulfite solution. It will be noted that formalin alone produced no change in the sulfite tubes while the bisulfite tubes showed some color. This does not correspond with the experiment on the Endo agar plates where formalin alone produced a deep color on the agar which had been decolorized with sodium sulfite. Otherwise the two experiments check fairly well.

TABLE 5. RESULTS OF ADDING ACID SOLUTIONS AND FORMALIN TO ALCOHOLIC SOLUTIONS OF BASIC FUCHSIN DECOLORIZED WITH SODIUM SULFITE AND SODIUM BISULFITE SOLUTIONS.

Solution	5% Lactic Acid	5% Acetic Acid	5% HSO	5% HCl	Lactic+ formalin	Acetic+ formalin	HSO+ formalin	HCl+ formalin	Formalin
Fuchsin bisulfite solution	—	—	—	—	++++	++++	++++	++++	++
Fuchsin sulfite solution	—	—	—	—	++	+++	++++	++++	—

CONCLUSIONS

The results of these various tests seemed to show that the hydrogen ion concentration does have a decided effect on the restoration of color in Endo agar, minute amounts of weak acid bringing about a marked change in color while small amounts of an alkaline solution decolorize the fuchsin more or less promptly. When tests were made on Endo agar of different reactions this fact held true. The more acid the medium the more difficult or impossible it became to decolorize the fuchsin completely. Color diffusion occurs more promptly and to a greater extent in a medium which is neutral or slightly acid.

It would also seem from these results that the conclusion reached by DeBord was more or less correct, that the reaction brought about in Endo agar by the colon organism is an acid aldehyde reaction, but that it apparently requires but a minute amount of acid when formaldehyde is present to stimulate this reaction.

In preparing Endo agar for culture work on the colon group of bacteria, alcoholic solutions of fuchsin give more satisfactory results than aqueous solutions, and French's test should be expanded to include alcoholic as well as aqueous solutions of basic fuchsin.

It also seems evident that sodium sulfite is on the whole a more satisfactory decolorizing agent than sodium bisulfite, especially in connection with alcoholic solutions of fuchsin.

NOTES ON TECHNIC

THE PREPARATION OF SOLUTIONS OF BASIC DYES. In a recent investigation of the effect of heat upon aqueous solutions of auramin it was shown by Holmes and Darling[1] that the dye underwent hydrolysis readily with the formation of tetramethyl-p-diamino-benzophenone and ammonium chloride. The reaction rate was measurable even at 30°C. and increased rapidly with increase in temperature. The accelerating influence of acidity was also decided. It was found, for example, that 50% decomposition of dye resulted in neutral aqueous solutions containing 0.2% dye at 70°C. in 12.5 hours. At the boiling point, the same degree of hydrolysis was obtained in 1.2 hours. At the latter temperature in solutions of which the acidity corresponded to 1.8 in the pH-scale an equivalent effect was obtained in about three minutes.

No definite data of a similar character appear to be available for other basic dyes. Their aqueous solutions are much more stable than that of auramin, and it is probable that relatively protracted boiling would result in but slight deterioration in some instances. With other basic dyes, however, among which may be specified the bismarck browns and the chrysoidines, it would probably prove decidedly deleterious.

In view of these circumstances dye manufacturers occasionally remind their customers that solutions of basic dyes should be made up and employed with as little resort to heating as may be necessary. Excessive heating is never necessary or beneficial and may prove decidedly injurious, particularly if other conditions are operative which may contribute to the instability of the dyes.

These facts are cited in the belief that they are probably unfamiliar to the majority of microscopists. The avoidance of all unnecessary heating is advocated as a judicious precaution in the preparation of solutions of basic dyes in general which are intended for application in biological staining. W. C. HOLMES.

A MODIFICATION OF THE CASARES-GIL FLAGELLA STAIN. The Casares-Gil technic for staining flagella is, perhaps, better known in recent literature as the Plimmer and Paine method. In 1921, S. G. Paine[2] published a paper in which he gave credit to the late Prof. H. G. Plimmer for having developed a new technic for flagella staining. The details of the method had not been fully worked out when Prof. Plimmer died, and after some further work Paine published his

[1] The Hydrolysis of Auramine, J. Amer. Chem. Soc., **46**, 2343. 1924.

[2] Plimmer, H. C., and Paine, S. G. A new method for the staining of bacterial flagella. J. Path. and Bact. **24**, 286–288. 1921.

paper. Altho Paine was unaware of the fact at the time of his publication, Prof. Plimmer had been following closely a technic developed by Casares-Gil and brought to the attention of bacteriologists by Galli-Valerio[2] in 1915. When distributing reprints of his paper, Paine attached to them a typewritten statement acknowledging the similarity between the two methods.

As given in Paine's paper, the stain is as follows:

(1) Mordant:

Tannic acid, 10 g.
Aluminium chloride (hydrated), 18 g.
Zinc chloride, 10 g.
Rosanilin hydrochloride, 1.5 g.
Alcohol 60%, 40 c.c.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 c.c. of the alcohol first, and then the rest slowly. This alcoholic solution may be kept several years. For use, dilute with four parts of water, filter off precipitate and collect filtrate on the slide allowing it to act for 60 seconds.

(2) Stain: Carbol fuchsin.

Quite by accident the writer discovered that preparations far superior to those made by the above technic were obtained if, in treating the films, a one-to-one water dilution of the mordant was used instead of one part to four of water as called for in the original formula. The only drawback to this modification is that filtration is more difficult; but the excellent quality of the preparations obtained in this manner would seem to justify its use.

Extensive studies were made of various organisms with excellent results. Flagella have been demonstrated clearly on various aerobic spore-formers, on three or four motile cocci, descriptions of which are to appear elsewhere, as well as upon various organisms of the prodigiosus type whose flagella are notoriously difficult to stain. Comparisons were made between the Casares-Gil technic and methods of Loeffler,[3] Shunk,[4] Kulp,[5] and Rosen.[6] All gave good results in some cases, but the former was the only technic by which satisfactory preparations were obtained with every organism investigated. —Lida M. Thatcher, Geneva, N. Y.

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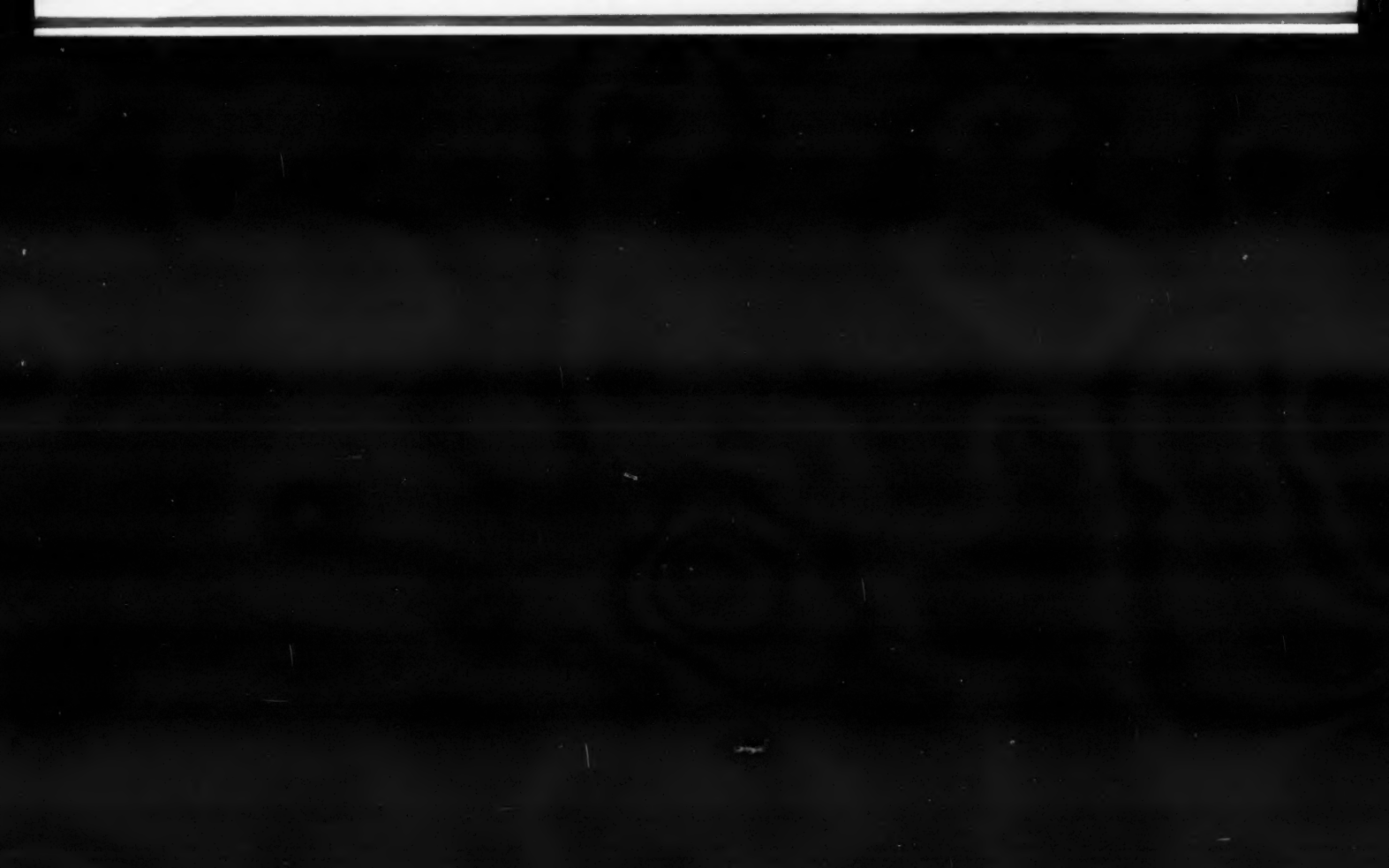
[2]Galli-Valerio, B. La méthode de Casares-Gil pour la coloration des cils des bactéries. Centbl. f. Bakt. 1 Ab. Or. **76**, 233–234. 1915.

[3]Loeffler, F. Weitere Untersuchungen über die Beizung und Färbung der Geisseln bei den Bakterien. Centbl. f. Bakt. **7**, 625–639. 1890.

[4]Shunk, I. V. A modification of Loeffler's flagella stain. J. Bact. **5**, 181–187. 1920.

[5]Kulp, W. L. A method for the staining of bacterial flagella. Stain Technology 1, 60–62. 1926.

[6]This technic has not yet been published. Details were obtained thru personal correspondence with the author.



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